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Exhibit L

The Mammary Gland as a Bioreactor: Expression, Processing, and Production of Recombinant Proteins

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A variety of transgenic animal species are being used to produce recombinant proteins. The general approach is to target the expression of the desired protein to the mammary gland using regulatory elements derived from a milk protein gene and then collect and purify the product from milk. Promoter sequences from a number of different milk protein genes have been used to target expression to the mammary gland, although significant problems remain with regard to achieving transgene expression levels consistent with commercial exploitation. The mammary gland appears to be capable of carrying out the complex posttranslational modifications, such as glycosylation and γ-carboxylation required for the biological activity and stability of specific proteins. Effective purification protocols have been established and products produced by this route have now entered clinical trials.

KEY WORDS: Transgenic; livestock; mammary gland; protein production.

INTRODUCTION

Human proteins have been used in medicine for many years. Most notable is the wide variety of plasma products supplied by the blood fractionation industry. These include immunoglobulin preparations, serum albumin, and concentrates of the clotting factors VIII and IX. In the past human proteins from sources other than blood have been extremely limited by supply. For example, until relatively recently, the only source of human growth hormone was pituitary glands procured from human cadavers.

Recombinant DNA technology has revolutionized the means of production of therapeutic proteins and no longer are supplies of a particular product limited by the supply of human material. Genes encoding a large number of human proteins have now been cloned, including insulin, growth hormone, protein C (hPC),[3] tissue plasminogen activator (htPA) and factors VII, VIII (hfVIII), and IX (hfIX). In some cases the expression of protein from these cloned genes has been accomplished in microorganisms, for example, in the production of human growth hormone and human insulin. For many proteins, however, microorganisms such as yeast and bacteria are not suitable because they require posttranslational modifications of their structure for biological activity or stability. Such modifications include the covalent addition of sugar residues, the chemical modification of certain amino acids and the specific cleavage of protein precursors. Bacteria and yeast are unable to carry out these modifications appropriately, but they are performed by mammalian cells. Consequently, there has been an intense effort to understand and optimize protein production in mammalian cell culture systems.

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[3] Abbreviations: beta-lactoglobulin (BLG); human alpha$_1$-antitrypsin (hα_1AT); locus control region (LCR); whey acidic protein (WAP); human factor VIII (hfVIII); human factor IX (hfIX); human protein C (hPC); human tissue plasminogen activator (htPA); chloramphenicol acetyl transferase (CAT); untranslated region (UTR).

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The culture of mammalian cells on a commercial scale is expensive and technically demanding. In 1982 an alternative to the production of proteins in cell culture was suggested by Palmiter and his colleagues (1). These workers described the production of transgenic mice carrying copies of the rat growth hormone gene. In these experiments the cloned rat gene had been introduced into the germline by the direct injection of DNA into the pronuclei of the fertilized mouse eggs. A proportion of the animals that developed from these eggs carried copies of the foreign DNA integrated at a site on one of the chromosomes. Prior to injection the rat sequences had been fused to regulatory DNA sequences from another gene, the metallothionein gene. This gene is expressed predominantly in the liver and, indeed, the metallothionein promoter sequences determined the site of expression. Large amounts of growth hormone were synthesized in the liver and the protein was secreted into the plasma, causing the animals to grow more rapidly and to a greater final size.

The plasma levels of the foreign protein were up to 100-fold higher than had been reported for growth hormone produced by genetically engineered bacteria or mammalian cells at that time and so the notion of producing therapeutic proteins in transgenic animals was born.

Work in transgenic mice, such as that described earlier for growth hormone, demonstrated the feasibility of targeting a particular protein to a specific tissue using the appropriate regulatory elements. We argued more than a decade ago that the mammary gland was the obvious tissue to target the expression of foreign proteins (2). Milk protein genes are expressed specifically and at high levels, and milk is collected easily and without detriment to the animal. In this paper I shall review the progress that has been made during the last decade in the development of this approach primarily for the production of human proteins, highlighting both the challenges and the successes.

PRODUCING TRANSGENIC ANIMALS

Pronuclear Injection. The basic techniques for producing transgenic animals were established in the mouse at the beginning of the 1980s (3). They involve the direct pronuclear injection of DNA sequences into the fertilized egg, followed by surgical implantation into the reproductive tract of a hormonally-primed recipient foster mother.

Transgenic mice have been used widely to assess the feasibility of many of the aspects of protein production in the mammary gland (see later). Indeed, the first paper describing the production of a recombinant human protein in milk, tissue plasminogen activator (htPA) was in transgenic mice (4). Mice, however, produce only small quantities of milk and, therefore, the realization of this approach has necessitated the development of techniques for generating large transgenic mammals. The pronuclear injection methodology has been adopted from the mouse, although some modifications have been required to take account of species differences. Successful gene transfer by this route has been described for all the major commercial livestock species including sheep (5), goats (6), pigs (7), and cows (8), as well as rabbits (9).

Working with larger domestic animals poses a number of technical and logistical problems. The recovery of suitable numbers of pronuclear eggs and their transfer following microinjection requires a large number of donor and recipient animals. The zygotes for injection may be obtained by superovulation of donor females or they maybe produced by the *in vitro* maturation and fertilization of oocytes obtained from the ovaries of slaughtered beasts. This eliminates the need for maintaining large numbers of donor animals and this approach is routinely employed in the generation of transgenic cattle (8). Microinjection of eggs from livestock is complicated by their relative opacity. In sheep and goats, careful microscopy using Nomarski differential interference optics is required to visualize the pronuclei. Bovine and porcine zygotes must be centrifuged to sediment the cytoplasmic lipid vesicles to enable visualization of the pronuclei although this does not appear to reduce embryo viability significantly (Fig. 1).

The efficiency of gene transfer, in terms of the number of eggs injected and transferred vs. the number of transgenic animals produced is usually between 1% and 2% (10). Once integrated in the germline the transgenes are usually, but not always, transmitted to the next generation. Rather disappointingly, there has been little improvement in the efficiency of this procedure during the last 10 years and this figure is similar to those given in the first reports describing the generation of transgenic livestock (5, 7). A number of workers have attempted to improve the overall efficiency of the injection procedure, for example, by devising cytoplasmic injection strategies, but with little success (11). Other groups have attempted to devise strategies to preselect embryos carrying the integrated transgene.

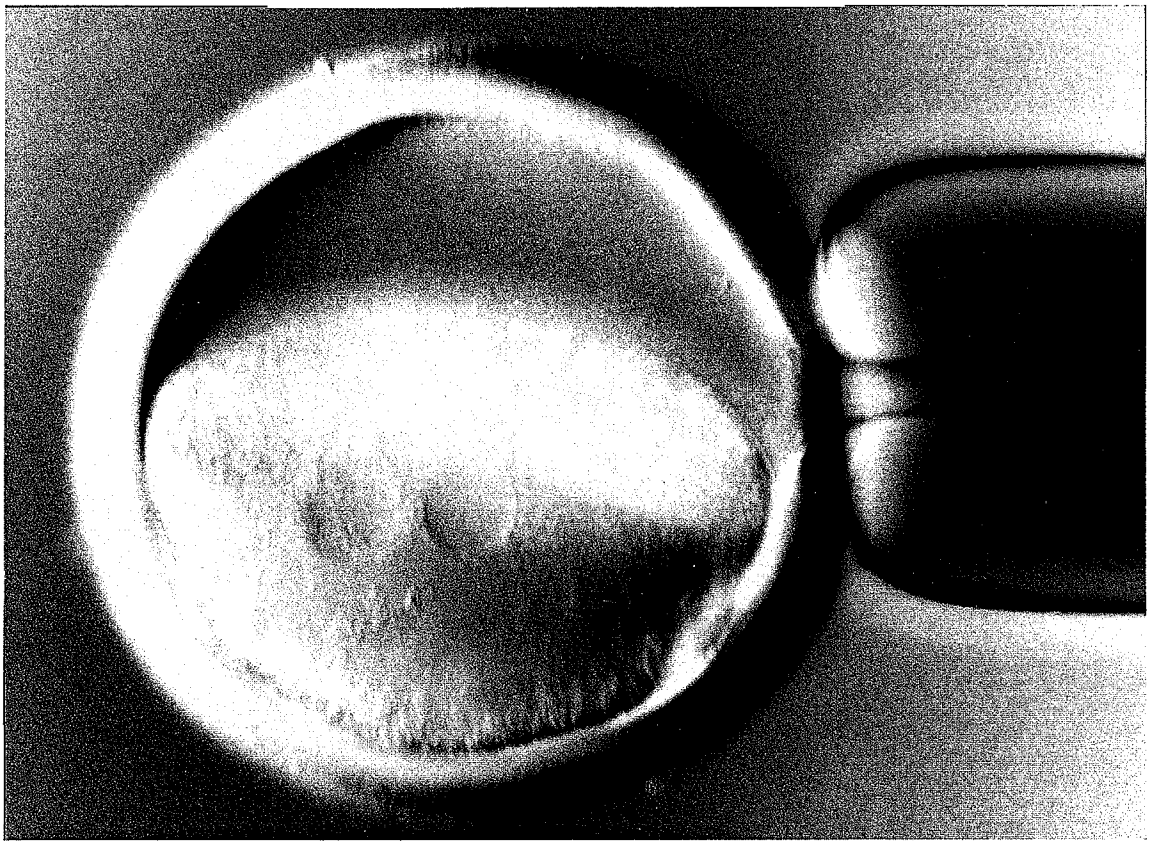

Fig. 1. Visualization of pronuclei. In livestock, visualization of the pronuclei is difficult because of the presence of dense lipid granules in the cytoplasm of the zygote. This problem can be resolved by centrifuging (12,000g/5 min) the zygotes prior to injection after which both male and female pronuclei become clearly visible. The holding pipette is on the right and the two pronuclei are clearly evident toward the top of the sedimented cytoplasm.

Transferring only positive embryos would dramatically reduce the number of recipient animals required in an experiment. However, working with the small amount of material available from embryo biopsies, it has proved impossible to reliably distinguish integrated from nonintegrated transgenes.

Cell-Based Transgenesis. A major bottleneck in the production of transgenic livestock has been the low efficiency of generating transgenic founders. The problem stems from the difficulty of having to work with the zygote as the cell into which the DNA is introduced. Zygotes can't be multiplied (as can conventional cells in culture) and they may only be cultured *in vitro* during the earliest stages of embryonic development. A radical improvement would be to accomplish the required genetic manipulation in conventionally cultured cells which could be used at a later stage to generate animals. Embryonic stem (ES) cells in mice are now widely used to manipulate the mouse genome. These are cells derived from the early embryo. They can be propagated in culture and, in the presence of the necessary growth factors, retain their totipotency. When injected back into a host blastocyst their descendants contribute to the tissues of the ensuing chimera including, most importantly, the germline. They are very tractable to DNA manipulation and have been used widely to target specific changes, such as gene deletions or replacements, in the genome. A selectable marker gene such as that encoding neomycin phosphotransferase *(neo)* is simultaneously introduced to allow selection of positive cells. In principle, then, ES cells would seem the ideal candidate from which to develop an alternative route to transgenesis in livestock. Despite intensive efforts, however, no validated ES cells (i.e., cells that will contribute to the germline) have been described for any species of livestock (12). Indeed, ES cells have not been described for any species other than the mouse, and in this species their generation appears to be limited to just a handful of inbred lines.

Until recently attempts to develop a cell-based system for transgenesis in livestock foundered on the failure to develop ES lines. Exciting, and largely unanticipated, developments in animal embryology, pioneered by Ian Wilmut and Keith Campbell at the Roslin Institute are, however, set to revolutionize transgenic technology in livestock. The key finding from these workers was that viable animals could be generated when nuclei from differentiated sheep cells that had been maintained in *in vitro* culture were transferred into enucleated oocytes (13). Contrary to dogma, the nuclei from these cells were not irreversibly determined but could be re-programmed to support full development to term and beyond. The breakthrough may be due to the use of quiescent cells in the G0 phase of the cell cycle as the nuclear donors, as it is thought that these nuclei may be more readily programmable. The first experiments were carried out using cultured cells derived from the early embryo (13). Subsequent experiments showed that other cell types including foetal fibroblasts and, most dramatically of all, adult mammary epithelial cells could be successfully used as the source of nuclei (14).

The ability to clone farm animals from cultured cells by nuclear transfer has profound implications for the generation and propagation of transgenic livestock. Recently, scientists at Roslin and PPL Therapeutics have described the production of transgenic sheep by nuclear transfer from transfected fetal fibroblasts (15). In these experiments the fetal fibroblasts derived from polled dorset sheep were cotransfected with a *neo* selectable marker gene and a gene encoding hfIX linked to regulatory elements from the sheep BLG gene. Two cloned transfectant cell lines as well as a population of *neo* resistant cells were used as donors in nuclear transfer experiments. Six transgenic lambs were liveborn; the three produced from the cloned cells contained both hfIX and *neo* transgenes whereas those from the uncloned population contained the marker gene only.

These experiments demonstrate for the first time the feasibility of using a cell-based route for transgenesis in large animals. Even in these early experiments this approach appears to be more efficient than pronuclear injection; it was estimated that less than half the animals that would have been required by conventional pronuclear injection were used (15). Nuclear transfer enables the cloning of identical copies of transgenic animals and so it may be possible to rapidly expand producer populations by using this approach rather than conventional breeding (Fig. 2). If gene targeting

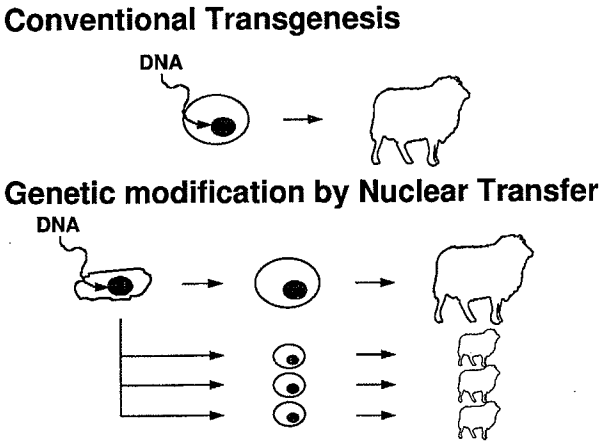

Fig. 2. Comparison of conventional transgenesis and cell based transgenesis using nuclear transfer. Using conventional transgenesis DNA is introduced into the 1-cell embryo. Transgenic status is usually determined after birth. In cell-based transgenesis the DNA is introduced into cultured cells using a selectable marker and positive cells selected prior to nuclear transfer into an enucleated oocyte (15). If cloned cells are used, then the transgenic status of the animals that are generated is guaranteed. Further nuclear transfer from the cells could generate a clonal population of transgenic animals. If gene targeting stategies can be adapted to this procedure, then the possibility of carrying out precise genetic manipulations in livestock is opened up.

by homologous recombination can be achieved in donor cells such as fibroblasts, then the way is opened up for making much more precise manipulations to the genome. Pronuclear injection gives the experimenter no control over the site or structure of the transgene integration site with major effects on the efficiency and reliability of expression (see later). Gene targeting would enable transgenes to be inserted as single copies at defined sites in the genome to avoid positional effects due to the site of integration and the tandem repeat nature of conventionally generated arrays (16). In some circumstances it may be appropriate to use endogenous promoters by targeting the coding sequences downstream, rather than introducing the conventional hybrid construct. Although nuclear transfer technology has been only proven and properly reported in sheep, there are already press reports of cattle cloned by nuclear transfer from cultured cells and it can only be a matter of time before this technology becomes more widely adopted

Choice of Species. Notwithstanding the route for transgenesis that can be used there are clear decisions that must be made regarding the choice of species for protein production. These relate to the quantity of protein product that will be required and the timescale

for production. Two key considerations are the volume of milk produced per lactation and the generation time. Thus rabbits produce approximately a liter of milk per lactation and can be bred many times during a single year and a doe can produce up to 10 liters of milk during this period. By contrast a cow can produce more than 10,000 liters per lactation. However, to generate a naturally lactating transgenic cow requires a minimum time period of 2.5 years (9 months gestation; 1 year to sexual maturity; 9 months gestation) and possibly up to 2 years longer if the original founder animal was a bull. Sheep and goats lie in between these extremes They both can produce several hundred liters of milk per lactation and the time to the first lactation is approximately 1.5 years. It is not surprising, therefore, that it is in these two species that commercial levels of protein production were the first to be described (Table I) and it is these two species that have formed the first producer populations for the relevant commercial enterprises now exploiting this technology. Pigs have also been proposed as possible producer animals; they can produce in excess of 100 liters of milk per lactation and the time to the first lactation is about 15 months. They are one of the more tractable large animal species in terms of conventional transgenesis but are not conventional dairy animals, and obtaining milk from them is difficult and unpredictable. Nevertheless, a number of complex proteins include protein including human protein C and human factor VIII have been expressed in swine milk (Table I).

TARGETING GENE EXPRESSION TO THE MAMMARY GLAND

Milk Protein Genes. Milk comprises a relatively small number of major proteins that are secreted specifically by the mammary gland. These proteins are grouped into two major classes, caseins and whey proteins. In cattle, for example, there are four caseins (αS1, αS2, β, and κ) and two whey proteins (BLG and α-lactalbumin). The genes encoding these proteins are single copy and are transcribed at high levels specifically in the mammary gland during pregnancy and lactation. A large number of milk protein genes from a variety of different species have been cloned and characterized (17). Some of these genes have been introduced into transgenic mice and their expression assessed in the mammary gland. In some cases very high levels of expression have been achieved. For example, for goat β-casein (18) and sheep BLG (19) levels in excess of 20 mg/ml have been reported (see also Ref. 20).

Promoters. The specificity and high levels of expression of endogenous milk protein genes in the mammary gland (as well as selected milk protein transgenes) indicated that regulatory elements from these genes could be used to direct the expression of foreign genes to this tissue. During the last 10 years a number of different milk protein gene promoters have been used to target expression of a variety of human therapeutic proteins to the mammary gland

Table I. Expression of Recombinant Human Proteins in the Milk of Transgenic Livestock

Species	Protein	Promoter	Expression	Ref.
Rabbit	hIL 2	rabbit β-casein	0.45 ug/ml	9
	hIGF-1	bovine αs_1-casein	1.0 mg/ml	59
	hα_1AT	goat β-casein	4.0 mg/ml	54
	hPC	ovine-BLG	0.7g mg/ml	54
Sheep	hfIX	ovine BLG	0.025 ng.ml	60
	hPC	ovine BLG	0.3 mg/ml	53,55
	hfibrinogen	ovine BLG	5.0 mg/ml	53,55
	hα_1AT	ovine BLG	35.0 mg/ml	25
Goat	htPA	goat β-casein	6.0 mg/ml	6
	hATIII	goat β-casen	14 mg/ml	54
	hα_1AT	goat β-casein	20 mg/ml	54
	Anti-cancer Mab	goat β-casein	10 mg/ml	54
Pig	hPC	mouse WAP	1 mg/ml	50
	hPC	ovine BLG	0.75 mg/ml	55
	hfVIII	mouse-WAP	3 mg/ml	51
Cattle	ha-lac	human a-lact.	2.4 mg/ml	55

[a] The maximum levels reported for each protein are tabulated against species and the promoter elements used to drive expression. Adapted from Ref. 53.

(20). Many of these experiments have been carried out using transgenic mice, which are often used as a model to test construct design prior to transfer to larger species. Overall, the results are equivocal; most of the promoters tested did target expression to the mammary gland to some degree but with wide variations between different experiments. Most appeared to exhibit specific expression in the mammary gland although examples of ectopic transgene expression have been reported (21). In some cases very high levels of expression of the target protein were reported. For example, Meade and co-workers (22) reported a 1–2 mg/ml concentration of urokinase using the bovine αS1 promoter and Archibald and co-workers (21) 7–8 mg/ml of human α1-antitrypsin (hα AT) using the ovine BLG promoter.

Construct Design. The large number of experiments carried out in transgenic mice and livestock clearly demonstrated the feasibility of this approach for the production of foreign proteins in the mammary gland. Nevertheless, there is still tremendous variability in the efficiency of transgene expression even when the same regulatory sequences are used.

In a large study we compared the expression levels of a number of different transgenes driven by the BLG promoter that either contained or lacked introns (23). The results were quite clear in that the transgenes containing most or all of their natural introns were expressed much more efficiently than their intronless counterparts; this was consistent with observations from other laboratories (24). For example, constructs comprising the BLG sequences fused to the cDNA sequences encoding hα$_1$AT were poorly, if at all, expressed. By contrast, a construct comprising the same BLG promoter elements used in the cDNA constructs but fused to a genomic minigene encoding human α$_1$AT was expressed very efficiently and mice expressing 7–8 mg/ml of the human protein were produced (21). This same construct was introduced into sheep; one founder animal exhibited expression at incredibly high levels—more than 30 mg/ml (25), clearly showing the feasibility of the approach for producing large amounts of a given human protein.

The identification of regulatory regions outside the conventional promoter region that enhance transgene expression, first exemplified in the β-globin gene cluster (26) and now termed locus control regions (LCRs) raises the question as to whether similar dominant regulatory elements are present at milk protein gene loci which could be incorporated into constructs to augment expression. At the time of writing, however, no milk protein LCRs have been described. In some cases, however, elements from other genes have been evaluated. The A element from the chicken lysozyme gene has been incorporated into a WAP transgene (27). In transgenic mice the WAP gene is expressed in only about 50% of the lines generated. Furthermore, its regulation during pregnancy and lactation is different from that of the endogenous gene and is quite variable between lines. By contrast, all 11 lines in which the WAP transgene was juxtaposed to the A elements expressed the transgene and most showed appropriate developmental regulation. However the same elements failed to improve expression when incorporated into a BLG-CAT construct (16).

Position Effects. Most transgenes are strongly influenced by their site of integration in the host chromosome. We have argued that the vicinity of an actively expressed milk protein gene might provide a site of integration permissive for the expression of a normally inefficiently expressed transgene. In mice this could be accomplished by using ES cells and this general approach has been exemplified recently by Bronson and co-workers (28), who showed that targeting a transgene into a defined site at the hypoxanthine phosphoribosyl transferase (HPRT) locus significantly improved the reliability of its expression. Gene targeting has yet to be demonstrated in livestock and, therefore, we chose an alternative approach that involves the co-integration of two transgenes at a single chromosomal site. In this approach poorly expressed BLG derived transgenes designed to express hα$_1$AT or hfIX were co-integrated with the efficiently expressing unmodified BLG transgene (29). This strategy resulted in significant improvement of the efficiency and frequency of expression of these constructs in the mammary gland. The "rescue" effect appears to require the functionality of the co-integrated BLG gene and BLG genes from which the promoter has been removed do not confer this effect (30).

Transgene Silencing. Recently, a number of laboratories have reported that transgenes are susceptible to potent silencing effects that manifest as a heterocellular or mosaic pattern of expression reminiscent of classical Position Effect Variegation observed originally in *Drosophila* (31). We have observed similar effects in the mammary gland and lines expressing the BLG transgene can exhibit highly variegated patterns of expression (32). The mechanism behind these silencing effects is not fully understood, but a critical feature appears to be the multicopy repeat nature of

transgenes and the existence of repeat sensitive transcriptional repression has been postulated (33).

Silencing may also be dependent on the nature of the transgene sequences themselves. Thus it has been known for many years that cDNA and prokaryotic reporter sequences are difficult to express in transgenic animals (see earlier). By co-injecting such cDNA constructs with the efficiently expressed BLG gene the frequency and level was improved. In an extension to this approach we linked the two genes together prior to injection to test whether we could improve the reliability of the approach (34). Enhanced reliability was not achieved, and to our surprise the main effect observed was the complete silencing of the BLG gene in the majority of the lines generated (Fig. 3). These experiments were done with both mammalian cDNA (hfIX) and a prokaryotic reporter (CAT) and both these sequences efficiently silenced the adjacent BLG gene, suggesting that such sequences can serve as active foci gene for repression in the genome.

RNA PROCESSING

Most efforts with regard to improving transgene expression in the mammary gland have focused on trying to improve transcription rates and, relatively speaking, little effort has gone into issues surrounding RNA processing. For the most part this is understandable as many of the basic mechanisms of mRNA metabolism such as capping, polyadenylation and export from the nucleus are carried out by all cells. Nevertheless, there are some processes that do vary between different cell types and to achieve efficient expression it may be necessary to optimize these in the long run.

Splicing. Consensus splice sites (35) are distributed throughout pre-mRNA sequences, yet only a specific subset of these are activated during splicing in the nucleus. The selection of splice sites in pre-RNA is poorly understand but is known to be determined by both *cis* and *trans*-acting factors. When hybrid constructs are transcribed quite novel pre-mRNAs are often generated, particularly if cDNA or minigenes are employed and this could lead to the activation of cryptic sites. Unfortunately, the rules for predicting the likelihood of a particular donor or acceptor site becoming activated are virtually nonexistent; in many ways it becomes a matter of trial and error, although experiments in appropriate cell lines may provide useful screening for aberrant splicing events (36).

Activation of cryptic splice sites can have disastrous effects. In our attempts to express hfIX in the milk of transgenic mice we achieved relatively high steady state mRNA levels of the hfIX cDNA transgene by co-integrating it with the unmodified BLG gene (29). Virtually no hfIX protein was detected in the milk of these animals, however. On closer inspection of the mRNA generated in these experiments it was found to be $\sim$ 450 nt shorter than predicted. Cloning by RT-PCR and sequencing of the transgene mRNA revealed a 462 bp deletion within the hfIX cDNA sequences encompassing the C terminal amino acids and 3′ UTR (37). The deletion was flanked by consensus donor and acceptor splice sites, strongly suggesting the activation of cryptic splice sites (Fig. 4). A transgene in which the 3′ acceptor site was removed was constructed and tested in transgenic mice. Pre-mRNA transcribed from this construct was no longer spliced aberrantly and relatively high levels of hfIX were secreted into the milk (37). Since hfIX cDNA appears to be correctly spliced when it is expressed in the liver (38) it seems likely that that the aberrant splicing observed in the mammary gland reflects differences in *trans*-acting splicing factors between these two tissues.

mRNA Stability. Although mRNA stability is a key feature in determining expression levels, the mechanisms that determine decay rates are only just beginning to be understood. At least three mechanisms mediate mRNA stability, poly A shortening, translational impairment and endonucleolytic cleavage (reviewed in Ref. 39). A large number of *trans*-acting factors that play a role in mRNA stability have been described whose functions vary from nucleolytic activity to more complex roles such as targeting mRNAs to specific degradative pathways. Some of these factors act globally while other are restricted to specific mRNA subsets. Interestingly, early work on the expression of casein genes suggested an important role for hormonally-mediated stabilisation of mRNA as a means of regulating expression levels in the mammary gland (40). *In vivo* in the lactating gland milk protein mRNAs exhibit very high steady state levels (5–10% total poly(A) mRNA in some cases) and it is tempting to speculate that mechanisms which prevent decay contribute to these levels. Little effort, however, has gone into this area with respect to improving transgene expression, even though a number of nucleotide elements have been described which apparently increase the cytoplasmic accumulation of eukaryotic mRNAs (41).

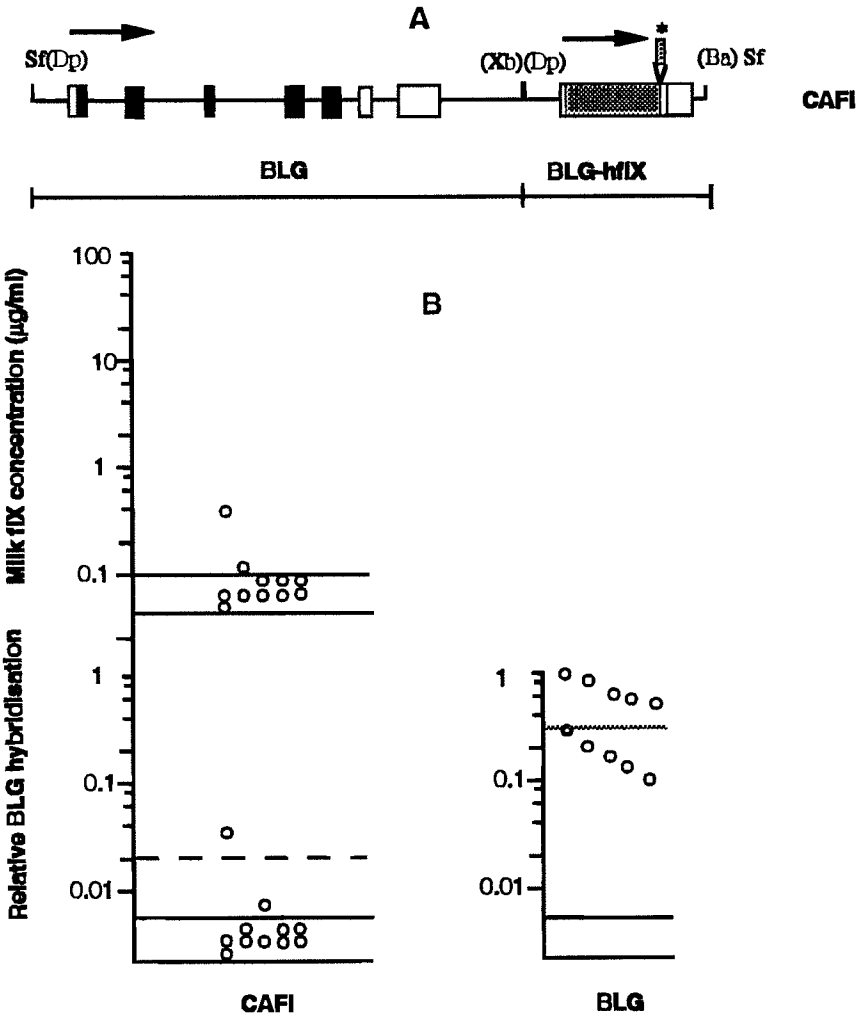

Fig. 3. Transgene silencing. (A) A two-transgene construct termed CAFI was built which comprises the BLG gene linked to a BLG-hfIX transgene and was introduced into transgenic mice. Open and full-shaded boxes, BLG exons; hatched box, hfIX cDNA sequences; line, BLG 5′ and 3′ flanking and intronic sequences. (B) Transgene expression profiles. The frequency and level of expression of various transgenes in the mammary gland are shown. Each circle represents an independently generated mouse line and usually represents the average level of expression determined in the G1 generation. Circles below the ordinate represent lines in which no transgene expression could be detected. In the transgenic lines carrying the CAFI construct (left) two/eleven lines showed low levels of fIX expression. Only two CAFI lines exhibited detectable BLG expression. By contrast when the same BLG gene used in the CAFI construct was introduced as a single trangene (right) all ten transgenic lines exhibited high levels of expresion. Adapted from Ref. 34.

Translational Control. mRNA translatability and stability are intimately linked and mRNAs in which translation is impaired often have reduced stability (39). For example, in addition to being a modulator of mRNA turnover the poly (A) status of an mRNA species also determines its translational efficiency. Most of our understanding of translational control comes from work in model systems such as Xenopus oocytes, yeast and cultured cells and little of this information has been applied to improving gene expression in transgenic animals. mRNA translation is affected by the structure encompassing the AUG translational initiation codon (42) and, in some instances, it may be appropriate to modify these sequences to more closely

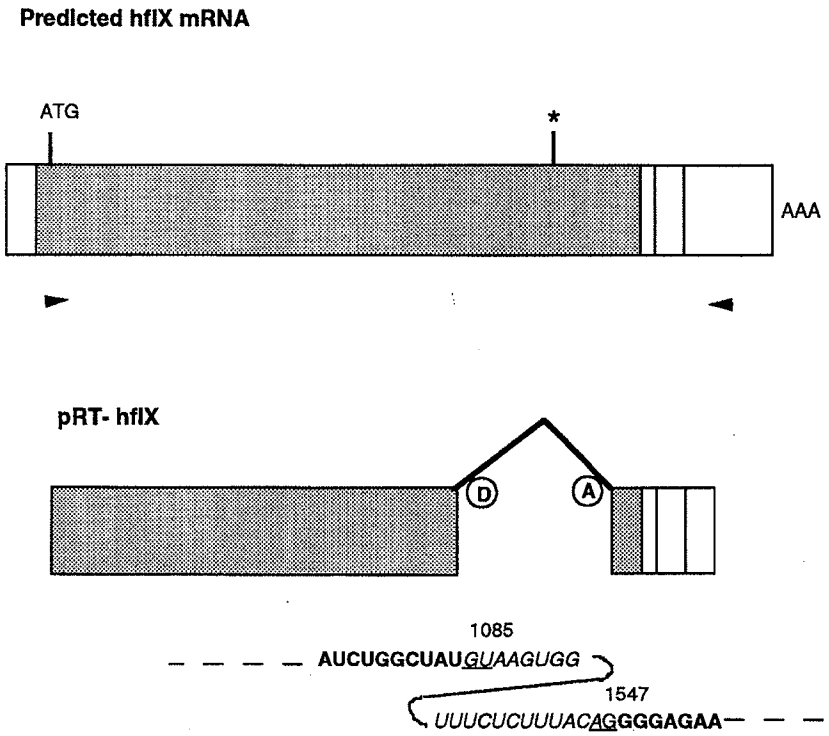

Fig. 4. Aberrant transgene mRNA splicing. hfIX cDNA sequences were targeted to the mammary gland using the BLG promoter. The predicted hfIX mRNA structure is shown. Northern blotting experiments indicated that the transgene mRNA was ~450 nt shorter than expected (29). These transcripts were amplifed by RT-PCR at the primer sites indicated. Sequencing the amplified product showed a 462 bp deletion at the 3′ end of the hfIX sequences. Inspection of the breakponts showed them to be flanked by consensus donor and acceptor splice sites indicating the activation of these cryptic splice sites in the mammary gland. Adapted from Ref. (37).

match the so-called "Kozak consensus." Other sequence elements which modulate translation have been identified within the coding region or 3′ untranslated regions (43) and, in principle, it may be possible to incorporate such elements into the transgene constructs to improve expression levels

POSTTRANSLATIONAL MODIFICATIONS

One of the principal reasons for using the mammary gland of transgenic animals for protein production is that many therapeutic proteins require posttranslational modifications for biological activity and/or stability which nonmammalian cells do not carry out correctly. Human proteins expressed in the mammary gland are being synthesized in a heterologous system, both in terms of species and cell type with unpredictable consequences for the nature and the degree of posttranslational modifications. Therefore, once acceptable expression levels of a protein have been achieved in milk, a critical next step is to characterize the posttranslational modifications as well as the overall biological activity of the protein.

Glycosylation. Glycosylation is a crucial posttranslational modification which can affect the solubility, stability, biological activity, and immunogenicity of many proteins. For example, glycoproteins containing high mannose or desialylated structures may have a short half-life in the circulation due to high mannose and asialoglycoprotein receptors in the liver.

Human γ-interferon contains two N-linked glycosylation sites at Asn 25 and Asn97. James *et al.* (44) demonstrated that γ-interferon expressed in the mammary gland of transgenic mice contained complex sialylated and fucosylated glycans at Asn 25 but high mannose structures at Asn 97 which could make such a protein susceptible to clearance by mannose receptors.

Denman and co-workers studied the glycosylation of a variant form of human tissue plasminogen activator expressed in the mammary gland of transgenic goats (45). They found lower levels of galactose, N-acetyl glucosamine and sialic acid in the transgenic protein as compared to the native protein. They also detected N-acetyl galactosamine residues which are not present in the native protein and could potentially lead to problems of immunogenicity. The bovine mammary gland is also known to process some N-linked glycoproteins aberrantly including the incorporation of terminal N-acetyl galactosamine residues and more complex branched mannose structures (46).

We have studied the glycosylation status of human hα_1AT produced in the mammary gland of transgenic mice (21). hα_1AT has three N-linked glycosylation sites and the glycosylation status of these is an important consideration for its pharmacokinetics. The material from transgenic mice exhibited biantennary and triantennary structures but exhibited a higher level of fucosylation than the human plasma derived material, although high mannose and hybrid structures were not detected (47). Given the variable and rather unpredictable nature of glycosylation, as illustrated by these examples, it seems likely that it will be absolutely essential to characterize in detail the glycan structures of each potential product prior to further development.

γ-Carboxylation. A number of human plasma proteins require the γ-carboxylation of certain glutamic acid residues for biological activity. The γ-carboxylated residues serve to bind calcium which is an essential cofactor for their activity. In the case of hfIX a cluster of 12 Glu residues near the N terminus must be γ-carboxylated for full biological activity (48). In our studies on the expression of hfIX in the mammary gland of transgenic mice we have used a monoclonal antibody specific for this calcium-binding Gla domain to purify the hfIX produced in the milk (37). This technique resulted in recovery of about half the hfIX from the starting material, all of which was shown to be fully active in a clotting assay. The mammary gland thus appears to be capable of carrying out the γ-carboxylation of hfIX quite efficiently. This finding is particularly gratifying, since γ-carboxylation has not been reported for any of the endogenous proteins made in the gland. Another γ-carboxylated protein, human protein C (hPC), has also been made in the milk of transgenic animals. In transgenic mice propeptide cleavage (see later) and γ-carboxylation were incomplete and only trace activity was detected (49). In transgenic pigs hPC was present in milk at levels between 0.5 and 1.0 mg/ml but only 30–60% of this material was fully active (50).

Other Modifications. Recently, the production of one of the largest and most complex plasma proteins, factor VIII, has been reported in the milk of transgenic swine (51). This protein requires N- and O-linked glycosylation and tyrosine sulfation for function. Although only quite low levels of the protein were obtained (3 μg/ml) it appeared to be correctly modified and exhibited biological activity.

Proteolytic Maturation. A number of potential therapeutic proteins including albumin and vonWillebrand's factor are first synthesized as inactive preproproteins which must be cleaved by specific endoproteases present in the endoplasmic reticulum and Golgi to yield the mature form of the protein. Functional human protein C (hPC) is converted from a single chain to a two chain molecule. When this protein was expressed in the milk of transgenic mice (49) or pigs (50) it was only partially processed and a mixture of the pro-single chain and the mature heavy and light chain was observed. In an elegant experiment, Drew *et al.* (52) devised an approach to enhance proteolytic maturation in the mammary gland. The serine protease furin has been shown to process a variety of proproteins in cultured cells. Double transgenic mice in which the expression of both hPC and furin were targeted to the mammary gland by the WAP promoter were generated. Co-expression of furin with hPC in the mammary gland resulted in efficient conversion of the pro-hPC precursor to the mature protein with cleavage occurring at the correct site. It should be noted that there may be significant species differences with regard to proteolytic processing capacity in the mammary gland. Thus although both mice and pigs do not appear to process hPC fully (in the absence of furin expression), transgenically derived material from sheep, expressed at levels up to 1 g/liter appears to be fully processed and is as active as plasma derived hPC in *in vitro* coagulation assays (53).

Multimeric Proteins. A number of potential protein products comprise two or more polypeptide chains encoded by separate genes. In these cases both genes must be expressed in the same cells in the mammary gland and the polypeptide chains processed, folded and appropriately assembled in the endoplasmic reticulum and Golgi apparatus prior to secretion. Monoclonal antibodies (Mabs) consist of separately encoded heavy and light chains and high levels of Mab expression (up to 5 mg/ml) have been reported in the milk of transgenic goats (54). Perhaps the most impressive

example in this regard is the high level of recombinant human fibrinogen which has been reported in sheep milk (53, 55). Fibrinogen comprises six polypeptide chains—dimeric α, β and γ chains. The three genes encoding the different chains were targeted to the mammary gland of transgenic sheep using BLG regulatory elements. Concentrations up to 5 g/liter were obtained in the milk, more than 1000-fold greater than was ever achieved in cell culture and the transgenic derived material appeared to be fully functional in a clotting assay.

DOWNSTREAM PROCESSING

A number of transgenic livestock expressing substantial amounts of human therapeutic proteins have now been produced (Table I). In many ways this is only just the beginning and many technical and regulatory hurdles must be crossed before a product that is suitable for administration to patients is realized.

Scale up. One of the advantages of pharmaceutical protein production in animals is that it offers a direct route for the scaling up of production by breeding producer populations. If the founder animal is female then the initial expression analysis can be carried out in this generation. Subsequent breeding from females has some limitations in terms of the progeny obtainable although using techniques such as transvaginal echoscopy to recover large numbers of immature oocytes *in vivo* (56), *in vitro* maturation and fertilization and embryo transfer can generate relatively large numbers of progeny. If the founder animal is male then daughters must be bred and expression analysis is delayed until their lactation in the next generation, although an early assessment of expression can be achieved by inducing lactation in prepubertal animals.

Breeding studies are important to determine the inheritance as well as the genetic and expression stability of the transgene locus. Transgenic sheep carrying a transgene targeting the expression of $h\alpha_1$AT to milk were bred (25, 26); of the six founders only four transmitted the transgene to the next generation. Of these four one female produced progeny with variable copy numbers of the transgene; this appeared to be the result of genetic rearrangement rather than the segregation of independently generated transgene integration sites (57). The three other founders all transmitted their transgene locus in as stable a fashion. From one of these (a male) a number of transgenic daughters were obtained. These all expressed $h\alpha_1$AT in milk at 13–16 g/liter in their first and subsequent three lactations. Similar levels of expression were obtained in the two subsequent generations and, interestingly, a homozygous female generated by breeding within the line expressed 37 g/liter of $h\alpha_1$AT; more than double the level of the heterozygous animals (53, 57).

Studies such as these demonstrate the feasibility of generating producer populations that stably transmit and express transgenes. The timelines for the generation of producer populations are, however, quite long. Given that it will be semen from the second generation that is used for breeding (to obviate problems of transmission or genetic instability) then, in sheep and goats it will be a minimum of 3 years before a lactating producer flock can be established; in cattle it will be more than 51/2 years before producers are on the ground. In the longer term cloning by nuclear transfer (14–16), particularly if it can be accomplished from expressing adults could have a significant impact on reducing these timelines.

During the establishment of the producer populations detailed characterization of the transgenic locus and the transgene product can be carried out. The animals must of course be housed in quarantined facilities and their health status continuously and individually monitored and a complete history of each individual maintained.

Purification. Because of the immunological consequences of administering animal proteins to humans, therapeutic products from the milk of transgenic animals must be purified to a very high degree. Furthermore the product must be pathogen free. The very high concentrations of foreign protein that can be achieved by transgenic approaches facilitates this process. PPL Therapeutics, a company set up to develop this technology have developed a purification process for $h\alpha_1$AT from transgenic sheep milk involving a combination of skimming, filtration, viral inactivation and chromatography steps. Validation of this process shows that the product produced has up to 23 logs of clearance of viral or prion proteins, should any be present (53). A Pilot Production Plant which processes hundreds of liters of sheep milk a week has been built and this currently produces more than 300 g of GMP grade $h\alpha_1$AT per week.

Clinical Trials. Extensive clinical trials are required for any new therapeutic products destined for human administration so that they can be guaranteed to be both safe and efficacious. Developed countries have regulatory authorities with statutory powers to ensure that new products are tested rigorously in both

preclinical and clinical trials before they can be used. Preclinical tests include biochemical, toxicity and pharmacokinetic properties as well as detailed information on the source and means of production. For protein products derived from the milk of transgenic animals this may include data on the complete sequence of the transgene, structure of the transgenic locus, specificity of transgene expression and the detailed genetic and health histories of the producer lines. These data are reviewed by the relevant authorities as a prelude to consent for the evaluation of the product in humans in clinical trials. Phase I trials are carried out on a small number of healthy volunteers and serve only to determine whether there are any adverse effects. If the phase I trial is successful then phase II trials are initiated to further evaluate safety and efficacy in patient groups and controls. Finally phase III trial are carried out in much larger patient groups and controls to fully evaluate the proposed use of the product. Only when these trials have been successfully completed will a license be issued allowing the manufacturer to sell the product commercially and, hopefully, recoup his investment.

Engaging in clinical trials is a very expensive procedure that invariably costs substantially more than all the other research and development costs put together and carries no guarantee of success. Presently there are two human protein products derived from the milk of transgenic animals that are negotiating this process. Genzyme Transgenics Ltd. is developing antithrombin III produced in the milk of transgenic goats for the treatment of clotting disorders (54) and PPL Therapeutics Ltd. is developing $h\alpha_1$AT as a treatment to ameliorate lung degeneration in cystic fibrosis (55). Both products successfully completed phase I and are now in phase II trials, so it will be sometime before these products reach the shelf.

CONCLUSIONS

It is just over 10 years ago that the idea of producing human proteins in the milk of transgenic livestock was conceived and the first experimental approaches initiated (2, 4, 5). In the intervening years substantial progress has been made. Many different human proteins have been targeted to the mammary gland in the mouse as well as in producer animals such as sheep or goats. In some cases extremely high levels of protein production have been achieved, far exceeding those obtainable by conventional batch cell fermentation, e.g., one transgenic ewe produced more than 30 g/liter $h\alpha_1$AT in her milk (25). This demonstration of the mammary gland's capacity to produce high levels of a recombinant protein was undoubtedly a milestone in the development of the technology. A key question from the outset was the gland's ability to carry out the necessary posttranslational modifications. By and large, it has risen to the task. Thus $h\alpha_1$AT produced in transgenic mice or sheep is quite similar (although not identical) to its human plasma derived counterpart (47, 53). However, the glycan structures synthesized by the mammary gland may not always be appropriate as evidenced by the branched mannose chains seen in γ-interferon made in mouse milk (44) and, more seriously, the N-acetylgalactosamine residues observed in htPA produced in goats and cows. (45, 46). The mammary gland also seems capable of carrying out complex processing such as γ-carboxylation and proteolytic maturation and the general impression is that it can carry these processes as efficiently, if not more so, than cells in culture. Again the efficiency of these processes may vary between species and target proteins making it important to evaluate carefully each recombinant protein produced and, if necessary, even consider augmenting the posttranslational processes themselves (52). In some cases protein production in the mammary gland has far exceeded our initial expectations. Certainly the demonstration that it can be used to produce high levels of complex multichain proteins such as fibrinogen which are fully active is a tour-de-force. Finally, the fact that two products are already in phase II clinical trials is a testament to the technology and those who have invested the time, effort and money during the last decade.

Notwithstanding these successes, there are certain aspects of the technology that have lagged behind. The fact that up until very recently the means and efficiency of generating transgenic livestock remained unchanged since the first reports of transgenic livestock (5, 7) has been disappointing. Secondly, despite the fact that our basic knowledge of the control of gene expression in the mammary gland has advanced substantially (e.g., see Ref. 58) this knowledge has been of little practical use when it comes to improving transgene expression. Other than using functionally well defined promoter segments and, where possible, including introns there are no robust rules for transgene design and the generation of high expressing lines is still rather a "hit and miss" procedure. Additional strategies have been tried with some success (29) but it remains a moot point

whether there can be a generic solution to improving transgene expression when variables such as the site and structure of the integration locus are outside the investigator's control. Adopting a cell based approach to transgenesis may solve some of these problems. Schneike *et al.* (15) have already shown that nuclear transfer from fibroblasts can be used as a route for transgenesis and, even at this early stage, appears to be more efficient than conventional pronuclear injection in terms of the number of animals required. In the future cloning of transgenic founders may be used to generate and expand producer populations and this could have a very positive impact in terms of cost and timescale. Establishment of gene targeting will enable transgenes to be inserted in a precise manner at defined sites and the expectation is that this may yield more reliably expressing animals. At this time, however, it is not yet clear which sites in the genome should be targeted and, for example, whether the same site would be suitable for different transgenes. Nevertheless, the path ahead is perhaps clearer than it was a year ago and there are evident opportunities to develop and improve this approach for the production of human proteins in livestock.

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